Arabidopsis* GOLDEN2-LIKE (GLK) transcription factors activate jasmonic acid (JA)-dependent disease susceptibility to the biotrophic pathogen *Hyaloperonospora arabidopsidis*, as well as JA-independent plant immunity against the necrotrophic pathogen *Botrytis cinerea

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SUMMARY

Arabidopsis thaliana GOLDEN2-LIKE (GLK1 and 2) transcription factors regulate chloroplast development in a redundant manner. Overexpression of *AtGLK1* (35S:*AtGLK1*) in *Arabidopsis* also confers resistance to the cereal pathogen *Fusarium graminearum*. To further elucidate the role of GLK transcription factors in plant defence, the *Arabidopsis glk1 glk2* double-mutant and 35S:*AtGLK1* plants were challenged with the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2. Compared with *Col-0*, *glk1 glk2* plants were highly resistant to *Hpa* Noco2, whereas 35S:*AtGLK1* plants showed enhanced susceptibility to this pathogen. Genetic studies suggested that *AtGLK*-mediated plant defence to *Hpa* Noco2 was partially dependent on salicylic acid (SA) accumulation, but independent of the SA signalling protein NONEXPRESSOR OF PATHOGENESIS-RELATED 1 (NPR1). Pretreatment with jasmonic acid (JA) dramatically reversed *Hpa* Noco2 resistance in the *glk1 glk2* double mutant, but only marginally affected the 35S:*AtGLK1* plants. In addition, overexpression of *AtGLK1* in the JA signalling mutant *coi1-16* did not increase susceptibility to *Hpa* Noco2. Together, our GLK gain-of-function and loss-of-function experiments suggest that GLK acts upstream of JA signalling in disease susceptibility to *Hpa* Noco2. In contrast, *glk1 glk2* plants were more susceptible to the necrotrophic fungal pathogen *Botrytis cinerea*, whereas 35S:*AtGLK1* plants exhibited heightened resistance which could be maintained in the absence of JA signalling. Together, the data reveal that *AtGLK1* is involved in JA-dependent susceptibility to the biotrophic pathogen *Hpa* Noco2 and in JA-independent resistance to the necrotrophic pathogen *B. cinerea*.

INTRODUCTION

The *GOLDEN2-LIKE* (*GLK*) genes are members of the GARP domain superfamily of transcription factors (TFs) (Riechmann *et al.*, 2000). *GLK* was originally identified as G2 in maize (Hall *et al.*, 1998) and was shown to be capable of transactivating the expression of a reporter gene in yeast (Rossini *et al.*, 2001). *Arabidopsis thaliana* *GLK1* (*AtGLK1*, GPRI1, At2g20570) and *GLK2* (*AtGLK2*, GPRI2, At5g44190) (Fitter *et al.*, 2002), also known as G-BOX binding proline-rich region interacting factors (GPRI) 1 and 2 (Tamai *et al.*, 2002), are similar to type B *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*), which are families of plant proteins related to prokaryotic response regulators of two-component systems (Riechmann *et al.*, 2000). The *AtGLK1* and *AtGLK2* genes have been shown to act redundantly to regulate chloroplast development (Fitter *et al.*, 2002; Yasumura *et al.*, 2005). The loss-of-function *glk1 glk2* double mutant is pale green in all photosynthetic tissues, with fewer thylakoid grana, whereas the loss of either *AtGLK1* or *AtGLK2* alone does not produce a pale green phenotype (Fitter *et al.*, 2002). The *AtGLK* proteins also act in a cell autonomous manner to coordinate and maintain the photosynthetic apparatus within individual cells (Waters *et al.*, 2008), and help to co-regulate and synchronize the expression of nuclear photosynthetic genes to optimize photosynthetic capacity in varying environmental and developmental conditions (Waters *et al.*, 2009). *AtGLKs* have also been shown to bind to promoters of several photosynthetic genes to upregulate the transcription of chloroplast genes (Waters *et al.*, 2009). More recently, it has been observed that *AtGLK2* can participate with *LONG HYPOCOTYL* (*HY5*) to achieve maximal effect in root greening in *Arabidopsis* (Kobayashi *et al.*, 2012; Nakamura *et al.*, 2009). In addition to binding to promoters, *AtGLK1* also interacts with proline-rich activation domains of G-BOX binding bZIP factors, suggesting that *GLKs* may regulate other related plant processes (Tamai *et al.*, 2002). Indeed, *AtGLK1* overexpression has been shown to play a role in pathogen response (Savitch *et al.*, 2007;

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Schreiber *et al.*, 2011), in nitrogen use efficiency (Gutiérrez *et al.*, 2008), in light- and brassinosteroid-induced chloroplast development (Nakamura *et al.*, 2009; Yu *et al.*, 2011) and in root hormonal signalling (Kobayashi *et al.*, 2012). Significantly, it has been observed recently that GLKs can interact with ANAC092 (ORE-1) to modulate leaf senescence in *Arabidopsis* (Rauf *et al.*, 2013).

Constitutive overexpression of *AtGLK1* (35S:*AtGLK1*) confers enhanced resistance to *Fusarium graminearum*, a nonhost fungal pathogen in *Arabidopsis* (Savitch *et al.*, 2007; Schreiber *et al.*, 2011), suggesting that *AtGLK1* may play a role in plant defence. In addition, an interactome network screening of plant pathogen effectors identified *AtGLK1* to interact with the pathogen effector AvrRpm1-like protein, although this interaction has not been confirmed directly (Mukhtar *et al.*, 2012). The *AtGLKs* can also interact with ANAC092 to modulate senescence (Rauf *et al.*, 2013), and with the involvement of ANAC092 in jasmonic acid (JA)/ethylene (ET) signalling (Bu *et al.*, 2008) and in disease resistance (Al-Daoud and Cameron, 2011). It is possible that GLKs may also be involved in responses to pathogen attack. To further elucidate the roles of *AtGLK1* in plant pathogen responses, we used the ectopic overexpression of *AtGLK1* (35S:*AtGLK1*) and the double knockout of *AtGLK1* and *AtGLK2* (*glk1 glk2*) to study the responses to the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2, an obligate biotroph causing downy mildew in the *Arabidopsis* host plant, and to *Botrytis cinerea*, a necrotrophic pathogen to *Arabidopsis*. Studies have indicated that the salicylic acid (SA) pathway plays a vital role in plant defence against biotrophic and hemibiotrophic pathogens, including *Hpa* (reviewed by Bari and Jones, 2009; Glazebrook, 2005). In addition, SA and JA defence pathways operating against different pathogens can be mutually antagonistic (Spoel *et al.*, 2003; Van der Does *et al.*, 2013; van Wees *et al.*, 2003) or can operate in a synergistic fashion depending on the nature of the pathogen, thus underscoring the complexity of the signal transduction network involved in plant defence (Beckers and Spoel, 2006; Ferrari *et al.*, 2003; Kunkel and Brooks, 2002; Mur *et al.*, 2006; Schenk *et al.*, 2000). Therefore, we introduced SA and JA signalling mutations into *glk1 glk2* and 35S:*AtGLK1* plants to dissect the role of GLKs in plant defence. We showed that, compared with *Col-0*, the *glk1 glk2* double mutant exhibited elevated resistance to *Hpa* Noco2, whereas 35S:*AtGLK1* was highly susceptible. Epistasis analyses with genes involved in the SA and JA signalling pathways suggested that the *AtGLK1*-mediated defence to *Hpa* Noco2 was partially dependent on SA accumulation, but independent of *NONEXPRESSOR OF PATHOGENESIS-RELATED 1* (*NPR1*). The studies also indicated that *AtGLK1* requires the JA receptor gene *CORONATINE INSENSITIVE 1* (*COI1*) to promote disease susceptibility to *Hpa* Noco2. In contrast, 35S:*AtGLK1* plants exhibited enhanced resistance to the necrotrophic fungal pathogen *B. cinerea* which was independent of *COI1*.

RESULTS

Atglk1/glk2-induced resistance to *Hpa* Noco2 is partially dependent on SA accumulation, but independent of the SA signalling pathway

The *glk1 glk2* double mutant and the overexpression of *AtGLK1* (35S:*AtGLK1*) were evaluated for their response to *Hpa* Noco2 infection. Compared with *Col-0*, the *glk1 glk2* double-mutant plants exhibited a strong restriction to *Hpa* Noco2 growth (Fig. 1a). Plants with mutations in either *AtGLK1* (*glk1*) or *AtGLK2* (*glk2*) displayed similar levels of *Hpa* Noco2 growth to the wild-type *Col-0*, suggesting that the resistance to *Hpa* Noco2 is the result of synergistic effects of both *glk1* and *glk2* (Fig. S1, see Supporting Information). This is consistent with observations of the redundant roles of *AtGLK1* and *AtGLK2* in chloroplast development (Fitter *et al.*, 2002; Yasumura *et al.*, 2005). Resistance to *Hpa* Noco2 in *Arabidopsis* is known to be mediated through the SA signalling pathway (Donofrio and Delaney, 2001; Lawton *et al.*, 1995; Nawrath and Métraux, 1999; Sanchez *et al.*, 2012), and this was verified here by enhanced susceptibility of *Col-0* plants expressing the bacterial enzyme salicylate hydroxylase (*NahG*), which catalyses SA to catechol (Fig. 1a). As the *glk1 glk2* double-mutant plants were highly resistant to *Hpa* Noco2, we were interested to determine whether this was the result of constitutive activation of the SA signalling pathway. First, we measured the levels of SA in uninfected plants. The results showed a dramatic 50% reduction in the *glk1 glk2* double-mutant plants compared with either the *Col-0* or 35S:*AtGLK1* plants (Fig. 1b). After infection with *Hpa* Noco2, *PATHOGENESIS-RELATED 1* (*PR1*) (*At2g14610*) was significantly lower in the *glk1 glk2* plants compared with either *Col-0* or the 35S:*AtGLK1* plants (Fig. 1c). This suggests that SA signalling may not be a factor in the resistance to *Hpa* Noco2 in *glk1 glk2* plants. These observations were verified by the expression of *NahG* in the *glk1 glk2* double mutant (*glk1 glk2 NahG*) and by the introgression of the SA signalling mutant *npr1-1* into the *glk1 glk2* double-mutant plants (*glk1 glk2 npr1-1*). As expected, *NahG* transgenic plants were susceptible to *Hpa* Noco2 (Fig. 1d) and the introduction of *NahG* into the *glk1 glk2* double mutant (*glk1 glk2 NahG*) only partially rescued the *Hpa* Noco2 susceptibility of *NahG* plants (Fig. 1d). Similarly, introgression of the SA signalling mutant *npr1-1* into the *glk1 glk2* double-mutant plants (*glk1 glk2 npr1-1*) did not overcome the resistance observed in the *glk1 glk2* double-mutant plants (Fig. 1d). These observations confirmed that the resistance observed in the *glk1 glk2* mutant plants was only partially the result of SA accumulation, and not mediated through *NPR1*. Correspondingly, in the 35S:*AtGLK1* plants, genes involved in the SA signalling pathway, such as *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *PHYTOALEXIN DEFICIENT4* (*PAD4*), *ACCELERATED CELL DEATH6* (*ACD6*) and the SA biosynthesis enzyme *ISOCHORISMATE*

Fig. 1 Resistance to *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2 is negatively regulated by *AtGLK1* and *AtGLK2* and is *NPR1* independent. (a) One-week-old wild-type (WT) (*Col-0*), *glk1 glk2* double-mutant, 35S:*AtGLK1* and *NahG* plants were inoculated with $1 \times 10^5/\text{mL}$ *Hpa* Noco2 spores. At 7 days post-infection (dpi), the spores were quantified from three samples of 25–30 seedlings per sample. The data from one representative experiment are shown and the error bars represent the standard error (SE). Similar trends were observed in three independent biological replicates. (b) Quantification of salicylic acid (SA), jasmonic acid-isoleucine (JA-Ile) and JA in 10-day-old seedlings of the WT (*Col-0*), *glk1 glk2* double mutant and 35S:*AtGLK1*. Analyses were performed on three separate biological samples and error bars represent SE. * and ** indicate significant differences between the WT and mutants at $P < 0.01$ and $P < 0.05$, respectively (Student's *t*-test). (c) *PATHOGENESIS-RELATED 1* (*PR1*) transcript levels relative to *UBIQUITIN 5* (*UBQ5*) were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) from RNA isolated at 72 h post-infection (hpi) with *Hpa* Noco2. Similar trends in relative transcript levels were observed in two independent biological replicates. (d) Analyses show that the resistance to *Hpa* Noco2 in the *glk1 glk2* double mutant is partially dependent on SA accumulation, but independent of *NONEXPRESSOR OF PATHOGENESIS-RELATED 1* (*NPR1*). The experimental details are similar to (a), but included *npr1-1*, *glk1 glk2 npr1-1* and *glk1 glk2 NahG*. FW, fresh weight.

SYNTHASE 1 (*ICS1*) accumulated to higher levels compared with those in *Col-0* plants (Fig. 2). This suggested that the susceptible phenotype observed in the 35S:*AtGLK1* plants was not the result of impairments in SA biosynthesis or signalling.

Pretreatment with SA partially rescues 35S:*AtGLK1* susceptibility to *Hpa* Noco2

Pretreatment with SA has been used to induce resistance against biotrophs, including *Hpa* Noco2 (Kunkel and Brooks, 2002; Ryals *et al.*, 1996). Accordingly, pretreatment with SA conferred increased resistance to *Hpa* Noco2 in *Col-0* plants, whereas *npr1-1* plants, which are defective in SA signalling, did not respond to SA treatment (Fig. 3a). A similar pretreatment of 35S:*AtGLK1* plants with SA only marginally reduced the susceptibility to *Hpa* Noco2 (Fig. 3a). Interestingly, pretreatment also reduced resistance in *glk1 glk2* plants, but these plants were still considerably more resistant than untreated *Col-0* plants. Together, these results suggested that the activation of the SA pathway led to partial rescue of the susceptible phenotype observed in the 35S:*AtGLK1* plants. The pretreatment, however, did not compromise the ability to express *PR1* in any of the mutant lines, and the expression was comparable with that of *Col-0* treated with SA (Fig. 3b). This suggested that the SA signalling pathway was functional in these mutants, but was not recruited to provide resistance to *Hpa* Noco2 (Fig. 3a).

Arabidopsis susceptibility to *Hpa* Noco2 requires both JA signalling and *AtGLK1*

Resistance to *Hpa* Noco2 is known to be achieved either by the activation of the SA pathway or, alternatively, by suppression of

Fig. 2 Transcript analyses of salicylic acid (SA) pathway genes on *Hyaloperonospora arabidopsidis* (Hpa) Noco2 infection. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of the indicated gene transcripts relative to *UBIQUITIN 5* (*UBQ5*) as internal standard at 72 h post-infection (hpi) with Hpa Noco2. (a) *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) transcripts; (b) *PHYTOALEXIN DEFICIENT4* (*PAD4*) transcripts; (c) *ISOCORISMATE SYNTHASE 1* (*ICS1*) transcripts; (d) *ACCELERATED CELL DEATH6* (*ACD6*) transcripts. The data are representative of one experiment. Similar trends in relative transcript levels were obtained from three independent biological replicates. WT, wild-type.

Fig. 3 Pretreatment with salicylic acid (SA) only marginally affects *Hyaloperonospora arabidopsidis* (Hpa) Noco2 susceptibility in 35S:AtGLK1 plants. (a) One-week-old seedlings were misted with either sterile water + 0.01% Silwet-77, which served as a control (–SA), or 1 mM SA in water + 0.01% Silwet-77 (+SA). The seedlings were covered with a clear plastic dome for 24 h prior to spray inoculation with $1 \times 10^5/\text{mL}$ Hpa Noco2 spores. At 7 days post-infection (dpi), spores were quantified from three samples with 25–30 seedlings per sample. Data from a representative experiment are shown and the error bars represent the standard error (SE). Similar trends were observed in three independent biological replicates. FW, fresh weight. (b) RNA was isolated from the samples from (a) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed relative to *UBIQUITIN 5* (*UBQ5*). Similar trends in relative transcript levels were obtained from three independent biological replicates. *PR1*, *PATHOGENESIS-RELATED 1*; WT, wild-type.

Fig. 4 Susceptibility to *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2 is dependent on jasmonic acid (JA) signalling. One-week-old seedlings were misted with either sterile water + 0.01% Silwet-77, which served as a control (–JA), or 0.1 mM methyl jasmonate in water + 0.01% Silwet-77 (+JA). The seedlings were covered with a clear plastic dome for 24 h prior to spray inoculation with 1×10^5 /mL *Hpa* Noco2 spores. At 7 days post-infection (dpi), spores were quantified from three samples with 25–30 seedlings per sample. Data from one representative experiment are shown and the error bars represent the standard error (SE). Similar trends were observed in three independent biological replicates. FW, fresh weight; WT, wild-type.

the JA pathway (Argueso *et al.*, 2012; Clarke *et al.*, 1998; Lawton *et al.*, 1995; Li *et al.*, 2001; Massoud *et al.*, 2012; Murray *et al.*, 2002; Rairdan and Delaney, 2002). In the absence of evidence to support SA-mediated resistance in the *glk1 glk2* plants, we hypothesized that the JA signalling pathway may be inoperative or compromised in the *glk1 glk2* plants or, alternatively, the JA pathway is constitutively active in the 35S:AtGLK1 plants. To test this hypothesis, we pretreated both *glk1 glk2* and 35S:AtGLK1 plants with methyl jasmonate (MeJA) and observed for disease phenotypes on *Hpa* Noco2 infection. Pretreatments with 0.1 mM MeJA dramatically reversed the resistance to *Hpa* Noco2 in the *glk1 glk2* mutant plants (Fig. 4). The pretreatment, however, did not increase significantly the already susceptible 35S:AtGLK1 plants (Fig. 4). To obtain further insights into the role of GLKs in the JA signalling pathway, we crossed *coi1-16*, a mutant that does not bind JA or JA-isoleucine (Ile), into the *glk1 glk2* double-mutant and 35S:AtGLK1 plants. The *coi1-16* mutant was similar to *Col-0* plants in susceptibility and, as expected, did not respond to JA application to increase this susceptibility (Fig. 4). However, the introduction of *glk1 glk2* into the *coi1-16* mutant plants (*coi1-16 glk1 glk2*) completely restored the resistance to *Hpa* Noco2 to at least the level of the *glk1 glk2* mutant (Fig. 4). This suggested that

the activation of the JA pathway by MeJA treatment was necessary for susceptibility and was mitigated by the presence of GLKs. This was further supported by the observation that plants overexpressing *AtGLK1*, which were susceptible with or without MeJA pretreatment, were completely resistant in the *coi1-16* mutant background (35S:AtGLK1 *coi1-16*), and indicated that *AtGLK1* and JA/JA-Ile perception through *COI1* were both necessary for susceptibility to *Hpa* Noco2 (Fig. 4). Measurements of JA and JA-Ile showed no significant differences between the *Col-0* and 35S:AtGLK1 plants, and further supported the notion that the integration of GLKs and the JA signalling pathway was required to observe a susceptible phenotype against the *Hpa* Noco2 pathogen (Fig. 1b).

AtGLK1 confers resistance to *B. cinerea* independent of JA signalling

To corroborate our findings that *AtGLK1*, in addition to JA signalling, may be an integral component in plant defence, we employed another pathosystem that requires JA signalling for defence. Unlike, *Hpa* Noco2, resistance to the necrotroph *B. cinerea* in *Arabidopsis* is known to be mediated through the JA signalling pathway (Glazebrook, 2005; Grant and Jones, 2009). To determine whether genetic manipulation of GLKs can alter responses to this pathogen, excised leaves from the *glk1 glk2* double-mutant and 35S:AtGLK1 plants were infected with *B. cinerea*. We observed that 35S:AtGLK1 plants were considerably more resistant than *Col-0* plants to *B. cinerea* and that the resistance was maintained even after 5 days (Fig. 5a–c). In contrast, *glk1 glk2* plants were significantly more susceptible than *Col-0* at 48 h after infection and the lesion continued to grow even at 5 days post-infection (dpi), similar to the *coi1-16* control plants (Fig. 5a–c). The *coi1-16 glk1 glk2* triple mutant was equally susceptible to the individual *coi1-16* and *glk1 glk2* mutants (Fig. 5a–c). In contrast, overexpression of *AtGLK1* in the *coi1-16* mutant background (*coi1-16 35S:AtGLK1*) resulted in a highly resistant phenotype similar to 35S:AtGLK1 plants (Fig. 5a–c). Furthermore, this resistance to *B. cinerea* was not accompanied by changes in transcript levels of JA signalling markers, such as *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), *Thionin 2.1* (*Thi2.1*), *PLANT DEFENSIN 1* and *2* (*PDF1* and *PDF2*) (compare *coi1-16 35S:AtGLK1* infected with *coi1-16* infected in Table S3, see Supporting Information). This suggested that, unlike the *Arabidopsis* response to the biotroph *Hpa* Noco2, overexpression of *AtGLK1* obviated the requirement of *COI1* in resistance to the necrotroph *B. cinerea*. Conceivably, *AtGLK1* can function to facilitate resistance to *B. cinerea* either downstream or independent of *COI1*.

DISCUSSION

Previously, we have reported that constitutive overexpression of *AtGLK1* confers enhanced resistance to the necrotrophic fungal

Fig. 5 *AtGLK1* positively regulates resistance to *Botrytis cinerea*. Detached leaves from 4-week-old plants were inoculated with 6 μ L of *Botrytis cinerea* spores (5×10^5 spores/mL) and photographed at the indicated times. Lesion sizes were measured and quantified by ImageJ software (<http://www.nih.gov/ij>). Data represent the means $\pm$ standard deviation (SD) from 30 infected leaves. (a) 48 h post-infection (hpi); (b) 5 days post-infection (dpi); (c) lesion measurements at 48 hpi and 5 dpi. The data are representative of three independent biological experiments. Scale bar, 1 cm in (a) and (b). WT, wild-type.

pathogen *F. graminearum* in *Arabidopsis* (Savitch *et al.*, 2007; Schreiber *et al.*, 2011). The present study aimed to further elucidate the role of *AtGLK1* in disease resistance and to gain insights into plant responses to biotrophic and necrotrophic pathogens. SA biosynthesis and signalling have been demonstrated to be critical for resistance against plant pathogens (Argueso *et al.*, 2012; Bowling *et al.*, 1994; Clarke *et al.*, 1998, 2000; Delaney *et al.*, 1994; Glazebrook *et al.*, 1996; Li *et al.*, 2001; Murray *et al.*, 2002; Rairdan *et al.*, 2001; Vlot *et al.*, 2009; Wildermuth *et al.*, 2001). In addition, SA signalling has been identified to positively regulate resistance against *Hpa* (Lawton *et al.*, 1994; Sanchez *et al.*, 2012). Here, our observation that *glk1 glk2* double-mutant plants are highly resistant to *Hpa* Noco2, whereas 35S:AtGLK1 plants are highly susceptible, when compared with *Col-0* (Fig. 1a), suggests that *AtGLK1* is a negative regulator of resistance to *Hpa* Noco2. We have provided evidence that the *AtGLK1*-regulated

Arabidopsis response to *Hpa* Noco2 is partially dependent on SA accumulation, but independent of SA signalling. Further, we observed that the introduction of *NahG* into the *glk1 glk2* double mutant (*glk1 glk2 NahG*) only partially rescued the *Hpa* Noco2 susceptibility of *NahG* (Fig. 1d). The results with *NahG*, however, should be interpreted with caution as the expression of this gene has been shown to exhibit pleiotropic effects (Heck *et al.*, 2003; van Wees and Glazebrook, 2003). As the *glk1 glk2 npr1-1* triple mutant can rescue *npr1-1* susceptibility to *Hpa* Noco2 (Fig. 1d), we suggest that the regulation of *Hpa* Noco2 resistance in the *glk1 glk2* double mutant is independent of SA signalling. In conjunction with the observation that the expression of *PR1*, *EDS1*, *PAD4* and *ICS1* genes indicates activation of the SA pathway in the highly susceptible 35S:AtGLK1 plants (Fig. 2), the results suggest that *AtGLK1*-mediated defence responses to *Hpa* Noco2 are predominantly SA independent.

JA signalling and *AtGLK1* enhance susceptibility to *Hpa Noco2* in *Arabidopsis*

We have demonstrated that either overexpression of *AtGLK1* or activation of the JA pathway by MeJA in the *glk1 glk2* double-mutant plants leads to disease outcome (Fig. 4). Furthermore, SA pretreatment only partially rescues the disease phenotype in the *AtGLK1* plants (Fig. 3a). Together, these results suggest that overexpression of *GLK1* may antagonize SA-induced resistance against *Hpa Noco2*. When these results are juxtaposed with other observations, such as the resistant phenotype in *coi1 35S:AtGLK1* plants, it reinforces the notion that GLK may participate in JA–SA antagonism. JA–SA antagonism in the pathogen response is well established (Robert-Seilanianz *et al.*, 2011; Thaler *et al.*, 2012).

AtGLK1 is a positive regulator of defence against *B. cinerea* which is independent of JA signalling

Plants overexpressing *AtGLK1* (*35S:AtGLK1*) were highly resistant to *B. cinerea*. Conversely, *glk1 glk2* plants were highly susceptible (Fig. 5). It is generally recognized that resistance to biotrophs is mediated through SA signalling, whereas resistance to necrotrophs is mediated through JA signalling (Glazebrook, 2005). Plants impaired in JA signalling are more susceptible to necrotrophic fungal pathogens, including *B. cinerea* (Ferrari *et al.*, 2007; La Camera *et al.*, 2011; Rowe and Kliebenstein, 2008). As expected, *coi1-16* plants were highly susceptible to *B. cinerea* (Fig. 5). However, plants overexpressing *AtGLK1* in the *coi1-16* background (*coi1-16 35S:AtGLK1*) were highly resistant, suggesting that *AtGLK1*-induced resistance is independent of *COI1*. This *COI1* independence is further supported by observations of the lack of induced transcripts of JA signalling marker genes on infection with *B. cinerea* (Table S3; compare *coi1-16* and *coi1-16 35S:AtGLK1*). *COI1*-independent processes have been observed to be involved in resistance to necrotrophs, especially against *B. cinerea* (Birkenbihl *et al.*, 2012; Ferrari *et al.*, 2007; Laluk *et al.*, 2011; Pré *et al.*, 2008; Tang *et al.*, 2007) and soil-borne fungal pathogens (Ralhan *et al.*, 2012; Thatcher *et al.*, 2009). Coincidentally, *35S:AtGLK1* plants showed attenuated constitutive levels of *VSP2*, *Thi2.1* and *PDF* transcripts compared with *Col-0* and *glk1 glk2* (Table S3). The association of attenuated levels of JA signalling marker transcripts with enhanced resistance to *B. cinerea* has been reported for the *Atmyc2.2* mutant and the *anac019 anac055* double mutant (Anderson *et al.*, 2004; Bu *et al.*, 2008). Recently, *AtGLKs* have been reported to interact directly with ANAC092 (ORE-1) to modulate chloroplast degradation in senescence (Rauf *et al.*, 2013). It is therefore conceivable that *AtGLK1* can act independently and downstream of *COI1* by the suppression of JA signalling through interactions with *AtMYC2*, *ANAC019* and/or *ANAC055*, and thus play a critical role in plant defence.

A proposed model for *AtGLK1* in plant defence

The combined results of *Hpa Noco2* resistance in the absence of *AtGLKs* or the JA-receptor *COI1* and its reversibility by MeJA application suggest that *GLK1* functions upstream of JA signalling (Fig. 6). In the absence of *GLKs* (*glk1 glk2*) and on infection with *Hpa Noco2*, the JA pathway is compromised, resulting in resistance (possibly by the removal of JA antagonism to SA). On infection with *B. cinerea*, resistance is provided by the presence of *GLK1* in an as yet unknown mechanism which is independent of JA signalling. The requirement of *GLK* for resistance is evidenced by the susceptibility of *glk1 glk2* to *B. cinerea*, and the independence from JA signalling is demonstrated in the recovery of resistance in the *coi1-16* mutant by *GLK1* overexpression (*coi1-16 35S:AtGLK1*) and by the absence of the expression of target genes of JA signalling (Table S3). The mechanism of how *AtGLKs* influence plant defences is not known, but we could envisage a scenario in which ‘effectors’ from *Hpa Noco2* and *B. cinerea* could target *GLKs*, leading to JA-dependent susceptibility against the biotroph and JA-independent resistance against the necrotroph (Fig. 6). *AtGLK1* has been reported previously to interact with *AvrRpm1*, an effector from the bacterial pathogen (Mukhtar *et al.*, 2012).

Fig. 6 A proposed model for the role of *AtGLK1* in plant defence. *AtGLK* may be a potential target for virulence factors from *Hyaloperonospora arabidopsidis* (*Hpa*) *Noco2* and *Botrytis cinerea*. If GOLDEN2-LIKE (*GLK*) acts as a negative regulator of the jasmonic acid (JA) pathway, targeting it will activate this pathway and promote susceptibility to *Hpa Noco2* and resistance against the biotroph. The experimental evidence suggests that the promotion of susceptibility to *Hpa Noco2* requires the intact JA signalling pathway (open arrows). In contrast, this pathway is not required for resistance to *B. cinerea* (filled arrows). *COI1*, CORONATINE INSENSITIVE 1.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana (Col-0) single-knockout lines of *glk1* (At2g20570) and *glk2* (At5g44190) and the *glk1 glk2* double-knockout line, N9805, N9806, N9807, respectively, were obtained from the Nottingham Arabidopsis Stock Centre (NASC), Nottingham, UK. The Col-0 plants overexpressing *GLK1* (35S:AtGLK1) were constructed as described previously (Savitch *et al.*, 2007). *Arabidopsis thaliana* (Col-0), *glk1*, *glk2*, *glk1 glk2*, *npr1-1*, *glk1 glk2 npr1-1*, *glk1 glk2 NahG*, *NahG*, *coi1-16*, *coi1-16 glk1 glk2*, *coi1-16 35S:AtGLK1* and *35S:AtGLK1* seeds were surface sterilized and subjected to vernalization at 4 °C for 2 days. The seeds were planted in ProMix soil and grown in 16 h light and 8 h dark at 21 °C at 60% relative humidity and under a light intensity of 150 µmol photons/m²/s. Plants were fertilized every second week. Double and triple mutants were generated by hand crossing, and segregating F2 populations were polymerase chain reaction (PCR) genotyped to identify the homozygous double and triple mutants. The details are described in Figs S2–S5 (see Supporting Information).

PCR genotyping

PCR genotyping primers are listed in Table S1 (see Supporting Information) and the results of PCR genotyping of the mutants are shown in Figs S2–S5.

Pathogen infection, microscopy, photographs and imaging

Spores of *Hpa* strain Noco2 were freshly harvested from Col-0 seedlings in sterile water and adjusted to 10⁵/mL for all the infection experiments. One-week-old *Arabidopsis* seedlings were spray inoculated with *Hpa* Noco2 using a spray gun (Preval, Coal City, IL, USA), and the plants were covered with a clear plastic dome to maintain high humidity (>95%) and grown at 16 °C in 9 h light/15 h dark for 7 days in an Adaptis A1000 growth chamber (Conviron, Winnipeg, MB, Canada). At 7 dpi, *Hpa* Noco2 spores from infected plants were harvested in sterile water and quantified. For gene expression analyses, infected plants were harvested at 72 h post-infection (hpi) and quick frozen in liquid N₂ for RNA isolation. *Botrytis cinerea* strain B191 spores were harvested from potato dextrose agar (PDA) plates in sterile water, filtered through four layers of sterile cheesecloth and washed twice in sterile water. The spores were resuspended in potato dextrose broth (PDB) and diluted to a concentration of 5 × 10⁵/mL for pathogenicity tests. Detached leaves from 4-week-old plants were placed on three layers of moist sterile Whatman filter paper (Fisher Scientific, ON, Canada) inside a sterile plastic Petri dish. Leaves were inoculated individually with 6 µL of *B. cinerea* spores at the centre of the leaf and the Petri dish was sealed with surgical tape to maintain humidity. Plates were incubated in the dark for the first 12 h and then transferred to a growth chamber set at 21 °C and 16 h light/8 h dark. The diseased leaves were photographed at varying times to observe lesion spread. Lesion sizes were measured from the digital photographs using ImageJ software (<http://www.nih.gov>).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated by Trizol reagent (Sigma-Aldrich, Oakville, ON, Canada). Two micrograms of RNA were converted to cDNA by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). All of the qRT-PCRs were performed in triplicate by an Applied Biosystems Power SYBR Green Kit and the Applied Biosystems StepOne Plus Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems, Burlington, ON, Canada). For relative quantification, a standard curve for each primer set was created and *UBIQUITIN 5* (*UBQ5*) was used as internal control for standardization between samples. Data were analysed by StepOne 2.1 software. A list of the genes and primers used in qRT-PCR is given in Table S2 (see Supporting Information). Analyses of transcript level changes after *B. cinerea* infection (Table S3) were performed as follows: 4-week-old soil-grown plants were spray inoculated with either PDB (mock) or *B. cinerea* spores (2.5 × 10⁵/mL) in PDB; the plants were covered with a clear plastic dome to maintain high humidity and incubated in the dark for the first 12 h, and later transferred to a growth chamber at 21 °C with a 16-h light/8-h dark photoperiod. Leaf samples were harvested at 48 hpi and quick frozen in liquid N₂ for RNA isolation. Gene transcripts were analysed by qRT-PCR relative to *UBQ5* as internal standard. *P* < 0.05 was considered to be statistically significant.

Hormone analyses

Leaves (approximately 500 mg) from 10-day-old Col-0, *glk1 glk2* and 35S:AtGLK1 plants were harvested and immediately frozen in liquid nitrogen for SA, JA and JA-Ile analyses. The frozen fresh plant materials were extracted with methanol–water–glacial acetic acid (3 mL, 90:9:1, v/v/v) to which the internal standards were added (100 mL of solution containing acetonitrile–water 50:50, v/v, with 0.1% formic acid, containing 1 ng/mL of 3,4,5,6-*d*-4-hydroxybenzoic acid and 0.5 ng/mL of 2,2-*d*₂-JA). Following sonication (5 min) and incubation in an orbital shaker (4 °C, 5 min), the samples were centrifuged at 266 300 g for 10 min to pellet the debris. The supernatants were transferred to clean tubes and the pellets were resuspended in the extraction solution (2 mL), and the procedure was repeated. The supernatants from individual samples were combined with the initial extracted volume and the pellets were resuspended in methanol (1 mL). The extraction step was repeated a third time. After the supernatants had been combined, methanol was evaporated under reduced pressure. On ice, aqueous NaOH was added to each sample to obtain a basic pH (1 mL, 0.3 M), and was then further extracted with dichloromethane (3 mL). The aqueous layers were transferred to a clean tube, and the organic layer was re-extracted with aqueous NaOH (2 mL). On ice, the combined aqueous layers were acidified with 5% aqueous HCl (1 mL) and then extracted with a mixture of ethyl acetate and cyclohexane (1 mL, 1:1, v/v). The organic phases were collected and the aqueous phases were extracted a second time with the same mixture (0.5 mL). The organic fractions were pooled and the solvent was evaporated under a constant nitrogen stream. Prior to mass spectrometric analysis, the samples were reconstituted in a mixture of methanol and water (200 mL, 30:70, v/v) containing 0.1% formic acid, to which external standards were added (100 ng of 1,2,3,4,5,6-¹³C₆-2-hydroxybenzoic acid and 50 ng of 12,12-*d*₃-JA). SA and JA were purchased from Sigma-Aldrich; JA-Ile was pur-

chased from OlChemIm Ltd. (Olomouc, Czech Republic). The preparation of standards and analyses were carried out at the National Research Council of Canada, Saskatoon, Canada. Deuterated forms of the hormones which were used as internal standards, 2,2-*d*₂-JA and 12,12,12-*d*₃-JA, were prepared as described by Galka *et al.* (2005). The preparation of 1,2,3,4,5,6-¹³C₆-2-hydroxybenzoic acid has been presented elsewhere (P. W. S. Galka *et al.*, unpublished data, National Research Council, Sask, Canada). Calibration curves were created for all compounds of interest. In the absence of an appropriate deuterium- or ¹³C-labelled internal standard, JA-Ile was quantified using a calibration curve built on its response against *d*₃-JA. Quality control samples (QCs) were run alongside the tissue samples. For the SA/JA analysis, the analytical ACQUITY UPLC® HSS C18 column (2.1 mm × 100 mm, 1.8 µm; Waters Limited, ON, Canada) was used. The compounds were eluted from the column with a mixture of solvents comprising 1% formic acid in high-performance liquid chromatography (HPLC)-grade water (mobile phase A) and 1% formic acid in HPLC-grade methanol (mobile phase B), using a gradient mode. Analyses were performed by ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC/ESI-MS/MS) using a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and analyses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 *AtGLK1* and *AtGLK2* redundantly regulate the *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2 resistance in *Arabidopsis*. One-week-old seedlings were inoculated with $1 \times 10^5/\text{mL}$ *Hpa* Noco2 spores. At 7 days post-infection (dpi), the spores were quantified from three samples of 25–30 seedlings per sample. The data from one representative experiment are shown and the error bars represent the standard error (SE). Similar trends were observed in three independent biological replicates.

Fig. S2 Polymerase chain reaction (PCR) genotyping of the *glk1 glk2 NahG* plant. (A) The top gel shows the presence of the dSpm insertion in the *glk1.2* mutation detected with the 2bgs2 + Spm5 primer pair at 58 °C after 35 cycles of PCR, and the bottom gel shows the presence of the wild-type (WT) allele for *AtGLK1* detected with the GLK1-Promoter-F1 + AtGLK1-R primer pair at 58 °C after 35 cycles of PCR to confirm the homozygous status of the *glk1.2* mutation. The asterisk (*) represents nonspecific PCR

products. (B) The top gel shows the presence of the dSpm insertion in the *glk2.1* mutation detected with the Spm1 + AtGLK2-R primer pair at 62 °C + gradient primer annealing after 35 cycles of PCR, and the bottom gel shows the presence of the WT allele for *AtGLK2* detected with the AtGLK2-F + AtGLK2-R primer pair at 62 °C + gradient primer annealing with 35 cycles of PCR to confirm the homozygous status of the *glk2.1* mutation. PCR genotyping of *glk1.2* and *glk2.1* mutations was performed according to Fitter *et al.* (2002). (C) The presence of the NahG transgene was confirmed with the NahG-F + NahG-R primer pair at 58 °C after 35 cycles of PCR generating a 700-bp product. Aliquots of 5 µL of PCR products from each genotype were separated on a 2% (w/v) agarose gel.

Fig. S3 Polymerase chain reaction (PCR) genotyping of the *glk1 glk2 npr1-1* triple mutant. (A) The top gel shows the presence of the dSpm insertion in *glk1.2* detected with the 2bgs2 + Spm5 primer pair at 58 °C after 35 cycles of PCR, and the bottom gel shows the presence of the wild-type (WT) allele for *AtGLK1* detected with the GLK1-Promoter-F1 + AtGLK1-R primer pair at 58 °C after 35 cycles of PCR to confirm the homozygous status of the *glk1.2* mutation. The asterisk (*) indicates nonspecific PCR products. (B) The top gel shows the presence of the dSpm insertion in *glk2.1* detected with the Spm1 + AtGLK2-R primer pair at 62 °C + gradient primer annealing with 35 cycles of PCR, and the bottom gel shows the presence of the WT allele for *AtGLK2* detected with the AtGLK2-F + AtGLK2-R primer pair at 62 °C + gradient primer annealing after 35 cycles of PCR to confirm the homozygous status of the *glk2.1* mutation. Aliquots of 5 µL of PCR product from each genotype were separated on a 2% (w/v) agarose gel. PCR genotyping of *glk1.2* and *glk2.1* mutations was performed according to Fitter *et al.* (2002). (C) *NPR1* was amplified using the npr1-1 F + npr1-1R dCAPs primer pair at 58 °C and generated a 252-bp product, which was digested with *Nla*III (NEB) and separated on a 3% agarose gel. The PCR product was digested in the WT of the *NPR1* allele, but not in the *npr1-1* mutant allele.

Fig. S4 Polymerase chain reaction (PCR) genotyping of the *coi1-16 glk1 glk2* triple mutant. (A) The top gel shows the presence of the dSpm insertion in the *glk1.2* mutation detected with the 2bgs2 + Spm5 primer pair at 58 °C primer annealing with 35 cycles of PCR, and the bottom gel represents the presence of the wild-type (WT) allele for *GLK1* detected with the GLK1-Promoter-F1 + AtGLK1-R primer pair at 58 °C primer annealing with 35 cycles of PCR to confirm the homozygous status of the

glk1.2 mutation. The asterisk (*) represents nonspecific PCR products. (B) The top gel shows the presence of the dSpm insertion in the *glk2.1* mutation detected with the Spm1 + AtGLK2-R primer pair at 62 °C + gradient primer annealing with 35 cycles of PCR, and the bottom gel shows the presence of the WT allele for *GLK2* detected with the AtGLK2-F + AtGLK2-R primer pair at 62 °C + gradient primer annealing with 35 cycles of PCR to confirm the homozygous status of the *glk2.1* mutation. PCR genotyping of the *glk1.2* and *glk2.1* mutation was performed according to Fitter *et al.* (2002). (C) *COI1* in the WT was detected using the coi1-16 P2 + coi1-16 P4 primer pair at 64 °C primer annealing with 26 cycles of PCR. As *coi1-16* is a point mutation, the WT allele for *COI1* also generated a PCR product with the coi1-16 P2 + coi1-16 P3 primer pair; however, the coi1-16 P2 + coi1-16 P4 primer pair generated a PCR product only in the WT allele and not in the *coi1-16* mutant allele. PCR genotyping of the *coi1-16* mutant was performed according to Adams and Turner (2010). Aliquots of 5 µL of PCR products from each genotype were separated on a 2% (w/v) agarose gel.

Fig. S5 Polymerase chain reaction (PCR) genotyping of the *coi1-16 35S:AtGLK1* plant. (A) Presence of the genomic *GLK1* and cDNA of the inserted *AtGLK1* transgene. The *GLK1* genomic fragment was detected using the AtGLK1-F + GLK1 genomic-R primer pair at 58 °C after 35 cycles of PCR. (B) Detection of the *coi1-16* mutation using the coi1-16 P2 + coi1-16 P3 primer pair at 64 °C after 26 cycles of PCR. Aliquots of 5 µL of PCR products from each genotype were separated on a 2% (w/v) agarose gel. (C) *COI1* detected in the wild-type (WT) using the coi1-16 P2 + coi1-16 P4 primer pair at 64 °C after 26 cycles of PCR. Aliquots of 5 µL of PCR products from each genotype were separated on a 2% (w/v) agarose gel. As *coi1-16* is a point mutation, the WT allele for *COI1* also generated a PCR product with the coi1-16 P2 + coi1-16 P3 primer pair. However, the coi1-16 P2 + coi1-16 P4 primer pair generated PCR products only in the WT, but not in the *coi1-16* mutant alleles. PCR genotyping of the *coi1-16* mutant was performed according to Adams and Turner (2010).

Table S1 Primer combinations used to genotype mutant and transgenic plants.

Table S2 List of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers.

Table S3 Transcript levels of *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), *Thionin 2.1* (*Thi2.1*), *PLANT DEFENSIN 1* and *2* (*PDF1* and *PDF2*) after infection with *Botrytis cinerea*.